Bifunctional oligonucleotide probes synthesized using a novel CPG support are able to detect single base pair mutations

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Received March 17, 1989; Revised and Accepted August 11, 1989

ABSTRACT

A novel multifunctional controlled pore glass, MF-CPG (Fig. 1), has been synthesized and used to incorporate 3′ terminal primary aliphatic amines into synthetic oligonucleotides. MF-CPG consists of a unique succinic acid linking arm which possesses both a masked primary amine for label attachment and a dimethoxytrityl protected hydroxyl for nucleotide chain elongation. Using MF-CPG, we have devised a simple and convenient technique to attach non-radioactive labels to the 3′ terminus of oligonucleotides. Bifunctional probes can then be constructed by ^{32}P labeling the 5′ terminus with T4 kinase and gamma ^{32}P-ATP. Using such bifunctional oligonucleotide probes in conjunction with polymerase chain reaction (PCR) amplification, we were able to detect single base substitutions in a target segment of the human H-ras protooncogene employing either functionality. Our technique thus expands the potential applications for oligonucleotides as hybridization probes.

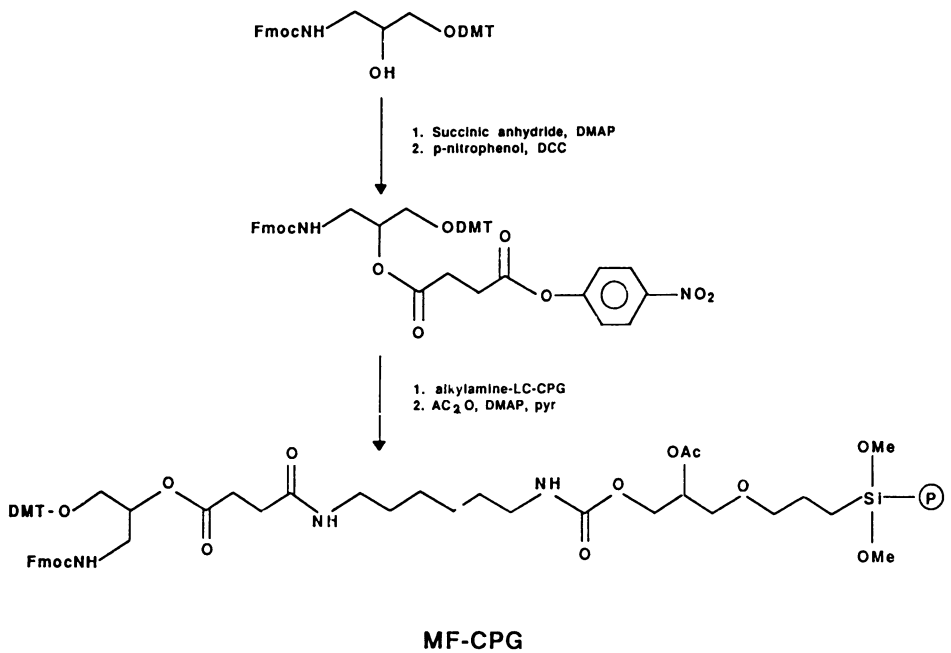
INTRODUCTION

Methods to covalently attach labels and reporter molecules to oligonucleotides have permitted their use as non-radioactive hybridization probes. New technologies in non-isotopic gene probes[1-9], DNA sequencing analysis[10-13], electron microscopy[14], and X-ray crystallography[12] have provided impetus for the development and improvement of such methods. As applications continue to emerge, more convenient oligonucleotide labeling techniques and reagents will be required.

Current methods to introduce chemical modifications into oligonucleotides employ special phosphoramidite reagents during solid phase synthesis. Attention has focused on the 5′ terminus and a number of protected amino-alkyl phosphoramidites have been reported[2-4,10,11,14,15] to incorporate a 5′ terminal aliphatic primary amine. Oligonucleotides modified by these reagents can be subsequently derivatized with fluorophores, biotin, and other molecules. Similarly, phosphoramidite reagents have also been described which incorporate a thiol functionality on the 5′ terminus[12,13,16].

Techniques modifying the 3′ terminus are inconvenient and tedious. Lemaitre *et al*[17,18] have described the attachment of a ribonucleoside to the 3′ terminus of an oligonucleotide using T4 RNA ligase. Terminal 3′ modification was achieved after periodate-oxidation of the ribose ring followed by reductive amination. Another procedure by Zuckermann *et al*[19] incorporates a 3′ terminal thiol group via solid phase oligonucleotide synthesis. Although this procedure is more efficient, it requires many synthetic steps and purifications.

Herein we report the synthesis and use of a novel multifunctional controlled pore glass, MF-CPG (Fig. 1), which introduces an aliphatic primary amine to the 3′ terminus of an oligonucleotide via solid phase synthesis. MF-CPG consists of a unique succinic acid linking arm which possesses both a masked primary amine for label attachment and a



MF-CPG

Figure 1. Synthetic Scheme for MF-CPG

dimethoxytrityl protected hydroxyl for nucleotide chain elongation. Any oligonucleotide sequence can be synthesized on MF-CPG using conventional cyanoethyl phosphoramidite protocols in automated DNA synthesizers. After the usual ammonium hydroxide treatment for cleavage and deprotection, the 3′ amino-modified oligonucleotide is obtained. The method is efficient and convenient, requiring no extra steps or reagents other than those used for normal oligonucleotide synthesis.

Using this MF-CPG based synthesis, we have constructed oligonucleotide probes which can be biotinylated at the 3′ terminus, and ^{32}P radiolabeled at the 5′ terminus. These bifunctional probes were able to distinguish a single base pair substitution in a target segment of the H-ras protooncogene using either a streptavidin-alkaline phosphatase detection system or autoradiography.

MATERIALS AND METHODS

Long chain alkylamine CPG was purchased from Pierce Chemical Co. Biotin-XX-NHS ester, PCR amplimers, and the Gene-tect Non-Isotopic Detection System were obtained from Clontech Laboratories, Inc. *Taq* polymerase was acquired from Perkin Elmer Cetus. HPLC was performed on a Rainin Rabbit HPX System using aquapore C8 reverse phase columns (Applied Biosystems, Inc.) for both preparative (100×10 mm) and analytical (30×4.6 mm) runs. A Biosearch Cyclone DNA synthesizer was used for oligonucleotide synthesis.

Preparation of MF-CPG

To a solution of N-Fmoc-O-DMT-3-amino-1,2-propanediol[21] (2.2 g, 3.5 mmol) and 4-dimethylaminopyridine (200 mg, 0.9 mmol) in anhydrous pyridine (12 ml) was added

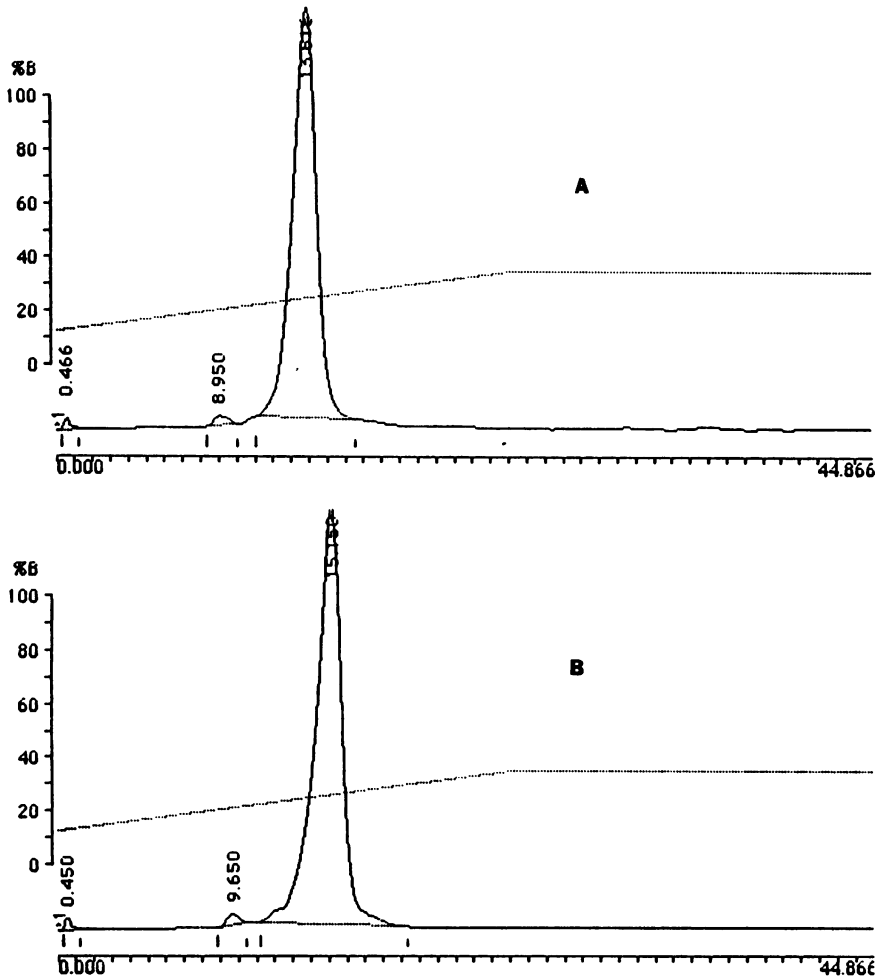


Figure 2. HPLC Chromatograms of Purified 3′ Biotinylated H-ras Oligonucleotide Probes. A RP-C8 analytical column was used with the following gradient system: A=0.1 M triethylammonium acetate (pH 7), B=40% acetonitrile in 0.1 M triethylammonium acetate; 12.5–35% B, 25 min, hold at 35% B. A: H-ras codon 13 wild type probe; B: H-ras codon 13 probe containing Asp mutation.

succinic anhydride (300 mg, 3 mmol). The reaction was stirred at room temperature for 17 hrs. The consumption of starting material was followed by TLC using methanol-dichloromethane (1:49) as the mobile phase. The mixture was diluted in ethyl acetate (100 ml), washed with 0.5 M sodium chloride (3×100 ml) and saturated sodium chloride (1× 100 ml), and dried over anh. sodium sulfate. After concentrating by rotary evaporation and drying under high vacuum (45°C), 1.74 g of a yellow solid was obtained.

The yellow solid was dissolved in dry dioxane (10 ml) containing anh. pyridine (0.5 ml) and p-nitrophenol (350 mg, 2.5 mmol). Dicyclohexylcarbodiimide (1.0 g, 4.8 mmol) was added and the mixture was stirred at ambient temperature. After a few minutes,

dicyclohexylurea began to precipitate. The reaction was monitored by TLC (methanol-dichloromethane, 1:9) and after 3 hrs the dicyclohexylurea was collected by filtration. Long chain alkylamine CPG (5.0 g) was suspended in the filtrate containing the p-nitrophenyl ester derivative, triethylamine (1.0 ml) was added, and the mixture was shaken overnight at room temperature. The derivatized support was copiously washed with dimethylformamide, methanol, and diethyl ether and dried *in vacuo*. Before capping the unreacted alkylamine groups, the loading capacity of the MF-CPG was assayed by determining the amount of dimethoxytrityl cation released upon treatment with perchloric acid according to published procedures[22].

Finally, capping of MF-CPG was achieved by treatment with acetic anhydride-pyridine-DMAP (10:90:1, v/v/w) for 1 hr. The support was thoroughly washed with methanol and diethyl ether and dried under high vacuum to give 4.95 g of MF-CPG. The capped MF-CPG gave a negative ninhydrin test[10].

Synthesis of 3' Biotinylated Oligonucleotide Probes

Two 3' amino-modified oligonucleotides, GGCGCCGGCGGTGTGGGCAA-X (H-ras, wild type) and GGCGCCGGCGATGTGGGCAA-X (H-ras, codon 13 Asp) [X = 3' primary amine modification], were synthesized using MP-CPG on a Biosearch Cyclone DNA synthesizer. Standard columns were packed with 1 μmol of MF-CPG and DNA synthesis was performed by suggested manufacturer protocols without any program changes. The coupling efficiency of the first nucleotide was determined by measuring the deprotected dimethoxytrityl cation concentration. Solid support cleavage and deprotection was accomplished with concentrated ammonium hydroxide.

Each crude amino-modified oligonucleotide was dissolved in 0.8 ml of 0.1 M $NaHCO_3/NaCO_3$ (pH 9). Biotin-XX-NHS ester in dimethylformamide (100 mg/ml, 0.25 ml) was added and the mixture was allowed to react for 16 hrs at room temperature. The biotinylated probes were purified on Sephadex G-25 columns (1×40 cm) and then by preparative HPLC. Analytical HPLC chromatograms of the purified 3' biotinylated probes are shown in Figure 2. The presence of biotin was confirmed by a p-methylaminocinnamaldehyde colorimetric test[23].

^{32}P 5' End Labeling of the Biotinylated Oligonucleotide Probes

The H-ras biotinylated probes were ^{32}P 5' end labeled using a modification of the method described by Berent *et al*[24]. 100 ng of the oligonucleotide probe were dissolved in 30 μl of distilled water and heated to 65°C for 3 minutes. The oligomers were then taken up to 50 μl of a reaction buffer which contained 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 μM DTT, 0.1 mM spermidine, 10 μl gamma ^{32}P-ATP (specific activity 6000 Ci/mM), and 15−20 units of T4 kinase. This reaction mixture was incubated at 37°C for 30 minutes followed by the addition of another 15−30 units of kinase and further incubation for 30 minutes. The labeled probe was then isolated using a G-25 Sephadex column.

PCR Amplification of the H-ras Protooncogenes

Amplification of genomic DNA by the polymerase chain reaction (PCR) has been previously described[1,20]. Two different sets of amplifying primers (amplimers) were used to amplify specific ras oncogene segments of genomic DNA. ATGACGGAATATAAGCTGGT (5' H-ras amplimer) and CTCTATAGTGGGGTCGTATT (3' H-ras amplimer) were used to amplify the region around codons 12 and 13 of the H-ras gene; ATGACTGAGTACAAACTGGT (5' N-ras amplimer) and CTCTATGGTGGGATCATATT (3' N-ras amplimer) were used to amplify the same codon region of the N-ras gene. 1 μg of genomic DNA was amplified in a 100 μl volume containing 50 mM KCl, 10 mM

Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.1% gelatin, 0.5 mM all four dNTPs, and 2.5 units Taq polymerase. The first amplification cycle included denaturation at 95°C for 5 min, annealing at 55°C for 2 min, and primer extension at 68°C for 2 minutes. The remaining 35 cycles were performed with 2 minute incubation periods at each temperature.

The generation of target DNA with a H-ras codon 13 Asp mutation was accomplished by the method described by Rochlitz *et al*[25]. In this reaction, the sequence of the 3′ amplifying oligomer has been noted above. The 5′ amplimer, however, encompasses the 20 nucleotide sequence at codon 12 and 13 of H-ras and contains a point mutation encoding a glycine to aspartate change in codon 13 (GGCGCCGGCGATGTGGGCAA). DNA generated through PCR amplification was used as target DNA in oligonucleotide hybridization analysis. In this manner, the amplified DNA incorporates the oligonucleotide with the codon 13 aspartate mutation.

Hybridization of Probe and Post Hybridization Washes with Tetramethylammonium Cloride (TMAC)

40 μl of the amplified samples were added to 80 μl of 0.4 N NaOH, and heated to 95°C for 2 minutes. The reaction mixtures were neutralized with 100 μl of 2 M Tris-HCl(pH 7.4), and the solution slotted onto Amersham Hybond nylon filter. The DNA was crosslinked onto the filter by UV radiation under conditions suggested by the manufacturer.

The slot blots were pre-hybridized for 2 hours at 37°C with 10−15 mls of 5× SSPE, 5× Denhardt's, 0.5% SDS, and 100 mM sodium pyrophosphate (pH 7.5). A labeled probe was then added to this solution to a final concentration of 5×10^6 cpm/ml and the filters were hybridized at 50°C for 4 to 12 hours. Following this incubation, the filters were washed once at room temperature with 6× SSC for 20 minutes, and twice at 61°C in 3 M TMAC, 50 mM Tris-HCl (pH 8), 2 mM EDTA, and 0.1% SDS. The filters were then washed once at room temperature with 6× SCC. Hybridization was detected both by autoradiography, and by colorimetric detection with a streptavidin-alkaline phosphatase conjugate (Clontech's Gene-tect System) on the same slot blot.

RESULTS

The preparation of MF-CPG is outlined in Figure 1. N-Fmoc-O-DMT-3-amino-1,2-propanediol[21] was first derivatized with succinic anhydride. The carboxyl group of the succinylated derivative was converted to a p-nitrophenyl ester and directly reacted with long chain alkylamine CPG to give MF-CPG. After capping, the loading capacity of MF-CPG was determined to be 27.8 μmol/g.

We constructed two bifunctional oligonucleotide probes using MF-CPG as follows. First, two 3′ amino-modified oligonucleotides, GGCGCCGGCGGTGTGGGCAA-X (H-ras, wild type) and GGCGCCGGCGATGTGGGCAA-X (H-ras, codon 13 Asp) [X = 3′ primary amine modification], were synthesized using MP-CPG. The coupling efficiency of the first nucleotide, which is indicative of 3′ primary amine incorporation, was >97% in both cases. After standard cleavage and deprotection with ammonium hydroxide, the crude 3′ amino-modified oligonucleotides were biotinylated with Biotin-XX-NHS ester. The long linking arm of Biotin-XX-NHS ester, a 14 atom spacer (XX) consisting of two e-aminocaproic acid moieties linked in series, was used to make the biotins more available for detection. After size exclusion on Sephadex G-25, the biotinylated oligonucleotides were purified by preparative HPLC on a C8 reverse phase column. Analytical HPLC chromatograms are shown in Fig. 2. The presence of biotin was confirmed by a p-methylamino-cinnamaldehyde colorimetric test[24]. Finally, the construction of the

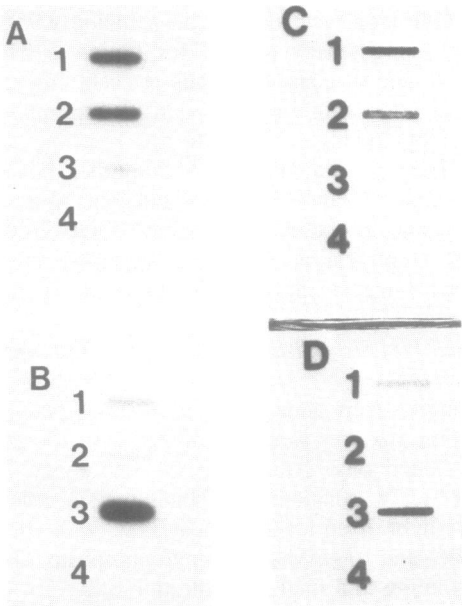


Figure 3. Detection of point mutations in the human H-ras gene using specific bifunctional oligonucleotide probes. DNA samples were PCR amplified and analyzed as described in the Materials and Methods. In all panels, slot 1 is DNA amplified around H-ras codon 13 from human placental DNA (GGT, glycine at codon 13), slot 2 is DNA amplified around H-ras codon 13 from mouse 3T3 cells transformed with a human H-ras gene bearing a glycine to valine mutation in H-ras codon 12 (GTT), slot 3 is human DNA amplified around H-ras codon 13 with a codon 13 aspartate mutation, and slot 4 is human placental DNA amplified around N-ras codons 12 and 13. Panel A: slot blot was hybridized with a ^{32}P-labeled oligonucleotide probe (20mer) specific for the wild type H-ras 13 sequence (GGT). The signal was detected by autoradiography. Panel B: slot blot was hybridized with a ^{32}P-labeled oligonucleotide probe (20mer) specific for an aspartate mutation in H-ras codon 13 (GAT). The signal was detected by autoradiography. Panel C: the same slot blot as in Panel A but the signal was detected colorimetrically using a streptavidin-alkaline phosphatase conjugate. Panel D: the same slot blot as in panel B but the signal was detected colorimetrically using a streptavidin-alkaline phosphatase conjugate.

bifunctional probes was completed by ^{32}P labeling of the 5′ ends with T4 kinase and gamma ^{32}P-ATP.

We assessed the applicability of these bifunctional oligonucleotide probes in detecting single base substitutions in the H-ras protooncogene. The probes used were specific either for the wild type H-ras sequence at codon 13 (GGT) or for the transforming mutant sequence at codon 13 (GAT) which substitutes aspartate for glycine. A 110 base pair segment which includes codon 13 of the H-ras gene was amplified from genomic DNA using the polymerase (PCR). By autoradiography (Fig. 3B), the codon 13 aspartate probe hybridized only with the amplified DNA carrying the H-ras codon 13 aspartate mutation and not with DNA from normal placenta, a transformed mouse fibroblast cell line (NIH-3T3) harboring a mutant human H-ras gene with a codon 12 valine substitution (GTT), or with placental DNA amplified in the region surrounding N-ras codons 12 and 13. Hybridization with the wild type codon 13 H-ras probe (Fig. 3A) however showed a signal only with amplified

DNA from normal placenta and the NIH3T3 cell line whose murine H-ras gene can also be amplified using our current amplimers (data not shown). Cross hybridization between the human and mouse wild type H-ras genes was unexpected but may indicate that the sequences probed appears are identical in the two species.

When the same filters were incubated with streptavidin-alkaline phosphatase and BCIP, colorimetric signals were seen over the same slots exhibiting a radiographic signal (Fig. 3C and 3D). Thus when used in conjunction with PCR these bifunctional probes are both sensitive and specific in detecting single base pair mismatches in target DNA. Furthermore, an oligonucleotide probe from a single MF-CPG preparation can be either biotinylated or radiolabeled without compromising hybridization sensitivity.

DISCUSSION

Single base substitutions in human genes are the cause or are strongly associated with a variety of human diseases such as the hemaglobinopathies[20,26] and cancer[1,27,28]. Previously, if no convenient restriction sites were altered by the base change, then the only recourse has been to clone and sequence the affected gene. Recently, PCR amplification of the DNA segment in question coupled with hybridization of specific oligonucleotide probes has allowed sequence determination without the need for molecular cloning. The applicability of the latter technique is dependent on the availability or versatile and inexpensive oligonucleotide probes. In this report, we have described a novel and simple method to synthesize 3′ labeled oligonucleotides.

In conventional solid phase DNA synthesis, the 3′ terminal nucleotide is pre-attached to the CPG support from the 3′ hydroxyl through a succinimic acid linking arm and the oligonucleotide is synthesized from the 5′ hydroxyl by repetitive cycles of chemical reactions. Therefore, an expedient strategy to introduce a 3′ primary aliphatic amine would be to replace the preattached nucleoside with a unique multifunctional linking arm. Using this strategy, we have constructed a multifunctional CPG, MF-CPG (Fig. 1) which transfers a primary amine to the 3′ terminus of a synthesized oligonucleotide without changing any chemistry or adding extra steps. MF-CPG possesses a uniquely engineered linking arm that complies with four important criteria. First, the linking arm is attached to the CPG through an ester functionality that is readily cleaved with ammonium hydroxide treatment. Second, the linking arm contains a masked primary aliphatic amine which is acid stabile and resistent to all the reagents used in normal oligonucleotide synthesis. Third, in addition to being acid stable, the amine protecting group is readily removed with ammonium hydroxide treatment. Fourth, the linking arm contains a dimethoxytrityl protected primary hydroxyl group for oligonucleotide chain elongation. Hence, the method is fully adaptable to commercial DNA synthesizers and is as easy as synthesizing normal oligonucleotides.

Since a reporter molecule now can be easily attached to the 3′ terminus of any oligonucleotide, both the 5′ and 3′ termini can be used to label the oligonucleotide. We have shown that such bifunctional oligonucleotide probes are both sensitive and specific in detecting single base substitutions in target DNA when used in conjunction with PCR. The sensitivity and specificity were the same regardless of the detection system; autoradiography or colorimetric detection with a streptavidin-alkaline phosphatase conjugate. The convenience of using MF-CPG to non-isotopically label an oligonucleotide at the 3′ terminus with subsequent ^{32}P labeling at the 5′ terminus makes this reagent an attractive alternative to current methods of functionalizing oligonucleotides. Thus, our technique expands the potential for applications employing functionalized oligonucleotides.

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REFERENCES

1. Liu, E., Hjelle, B., Morgan, R., Hecht, F., and Bishop, J.M. (1987) Nature **330**, 186–188.
2. Agrawal, S., Christodoulou, C., and Gait, M.J. (1986) Nucl. Acids Res. **14**, 6227–6245.
3. Connolly, B.A. (1987) Nucl. Acids Res. **15**, 3131–3139.
4. Jablonski, E., Moomaw, E.W., Tullis, R.H., and Ruth, J.L. (1986) Nucl. Acids Res. **14**, 6115–6128.
5. Haralambidis, J., Chai, M., and Tregear, G.W. (1987) Nucl. Acids Res. **15**, 4857–4876.
6. Li, P., Medon, P.P., Skingle, D.C., Lanser, J.A., and Symons, R.H. (1987) Nucl. Acids Res. **15**, 5275–5287.
7. Chu, B.C.F. and Orgel, L.E. (1985) DNA **4**, 327–311.
8. Kempe, T., Sundquist, W.I., Chow, F., and Hu, S. (1985) Nucleic Acids Res. **13**, 45–57.
9. Murasugi, A. and Wallace, R.B. (1984) DNA, **3**, 269–277.
10. Smith, L.M., Fung, S., Hunkapiller, M.W., Hunkapiller, T.J., and Hood, L.E. (1985) Nucl. Acids Res. **13**, 2399–2412.
11. Connell, C., Fung, S., Heiner, C., Bridgham, J., Chakerian, V., Heron, E., Jones, B., Menchen, S., Mordan, W., Raff, M., Recknor, M, Smith, L., Springer, J., Woo, S., and Hunkapiller, M. (1987) BioTechniques **5**, 342–345.
12. Sproat, B.S., Beijer, B., Rider, P., and Neuner, P. (1987) Nucl. Acids Res. **15**, 4837–4848.
13. Ansorge, W., Sproat, B., Stegemann, J., Schwager, C., and Zenke,M. (1987) Nucl. Acids Res. **15**, 4593–4602.
14. Sproat, B.S., Beijer, B., and Rider, P. (1987) Nucl. Acids Res. **15**, 6181–6196.
15. Sinha, N.D. and Cook, R.M. (1988) Nucleic Acids Res. **16**, 2659–2669.
16. Connolly, B.A. (1985) Nucleic Acids Res. **13**, 4484–4502.
17. Lemaitre, M., Bayard, B. and Lebleu, B. (1987) Proc. Nat. Acad. Sci. USA **84**, 648–652.
18. Lemaitre, M. Bisbal, C. , Bayard, B. amd Lebleu, B. (1987) Nucleosides and Nucleotides, **6**, 311–315.
19. Zuckermann, R., Corey, D. and Schultz, P. (1987) Nucleic Acids Res. **15**, 5305–5321.
20. Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., and Arnheim, N. (1985) Science **230**, 1350–1354.
21. Nelson, P.S., Sherman-Gold, R. and Leon, R. (1989) Nucleic Acids Res., **17**, 7179–7186.
22. Oligonucleotide Synthesis: A Practical Approach (1984) Gait, M.J., Ed., IRL Press, Oxford.
23. McCormick, D.B. and Roth, J.A. (1970) Methods Enzymol. **18**, 383–385.
24. Berent, S.L., Mahmoudi, M., Torczynski, R.M., Bragg, P.W., and Bollon, A.P. (1985) BioTechniques **3**, 208–220.
25. Rochlitz, C.F., Scott, G.K., Dodson, J.M. and Benz, C.C. (1988) DNA **7**, 515–519.
26. Embury, S.H., Scharf, S.J., Saiki, R.K., Gholson, M.A., Golbus, M., Arnheim, N., and Erlich, H.A. (1987) N. Engl. J. Med. **316**, 656–660.
27. Almoguera, C., Shibata, D., Forrester, K., Martin, J., Arnheim, N., and Perucho, M. (1988) Cell **53**, 549–554.
28. Rodenhuis, S., van de Wetering, M.L., Mooi, W.J., Evers, S.G., van Zandwijk, N., and Bos, J.L. (1987) N. Engl. J. Med. **317**, 929–935.
29. Bos, J.L., Fearon, E.R., Hamilton, S.R., Verlaan-de Vries, M., van Boom, J.H., van der Eb, A.J., and Vogelstein, B. (1987) Nature **327**, 293–297.

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